REQUIREMENTS FOR α-KETOGLUTARATE, FERROUS ION AND ASCORBATE BY COLLAGEN PROLINE HYDROXYLASE

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Received June 6, 1966

Collagen proline hydroxylase was first demonstrated in chick embryo microsomal fractions (Peterkofsky and Udenfriend, 1965) and was subsequently obtained in soluble form (Prockop and Juva, 1965a). In both preparations evidence for Fe^{++} involvement was obtained as well as requirements for heat-stable cofactors. Stimulation by ascorbic acid (Peterkofsky and Udenfriend, 1965) and potassium ions (Prockop and Juva, 1965b) was also reported. However, it was apparent from the reports of both laboratories that the cofactors listed above were not sufficient for activity. Recently, it has been possible to utilize partially purified dialyzed preparations of collagen proline hydroxylase from chick embryo to demonstrate absolute requirements for Fe^{++}, ascorbic acid and α-ketoglutarate.

Methods: Proline hydroxylase was prepared by homogenizing 8-day-old chick embryos in 0.25 M sucrose. The homogenate was centrifuged at 105,000 x g and the S-105 supernatant fraction

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was dialyzed. Following dialysis, 0.05 vol of 1 M, Tris-HCl buffer, pH 7.5 and 0.3 vol 5% streptomycin sulfate were added and after standing at 0° for 15 min, the mixture was centrifuged at 15,000 x g for 20 min. The supernatant solution was brought to 60% saturation with solid $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation, dissolved in a small vol of water and dialyzed to yield a partially purified enzyme solution.

Tritium labeled proline hydroxylase substrate was prepared as described by Hutton et al. (1966) by incubating 6 g of minced chick embryo in 8.5 ml modified Krebs-Ringer buffer in the presence of 500 μC 3,4-T-L-proline (T = tritium) and 1 mM α,α'-dipyridyl to inhibit hydroxylation. The substrate was extracted into cold 0.5 N acetic acid and dialyzed. Resulting substrate solutions were generally diluted to 2 mg/ml of protein containing 800,000 dpm/mg. Less than 1% of the radioactivity was in peptide-bound hydroxyproline.

Proline hydroxylase activity was assayed in a 2 ml vol containing the ingredients shown in the legends to the tables. The assay has been described in detail (Hutton et al., 1966) and depends upon the formation of THO (tritiated water) when the substrate, peptidyl-3,4-T-proline, is converted to peptidyl-3-T-hydroxyproline. One equivalent of THO is formed per equivalent of peptidyl-hydroxyproline synthesized. Reactions were stopped by adding 0.1 vol 50% TCA and THO was assayed after vacuum distillation. Results are presented as cpm THO, counting efficiency 8%.

Crystalline bovine liver L-glutamic acid dehydrogenase (GDH), was obtained from Sigma Chemical Co. At a concentration of α-ketoglutarate of 10^{-4}M, the quantity of GDH added to incubations described in Table II was sufficient to convert α-ketoglutarate to L-glutamate, at a rate of 13 μmoles/min.

Results and Discussion: Following dialysis, collagen proline hydroxylase preparations from chick embryos were found to be totally inactive. We have found, as was previously reported by Prockop and Juva (1965a), that addition of the dialysate is required for activity. Ascorbic acid and Fe^{++} were both stimulatory when added in addition to the dialysate, but had no effect when added to enzyme preparations individually or together in the absence of dialysate. A large number of substances were tested for their ability to replace the dialysate when added to incubation mixtures containing dialyzed enzyme, ascorbic acid and Fe^{++}. In this initial screening nucleotide phosphates, pyridine nucleotides, amino acids, vitamins and metal ions were found to be without activity. Of the Krebs cycle intermediates, only α-ketoglutarate and to a lesser extent oxaloacetate were capable of replacing the dialysate (Table I). The apparent K_m for α-ketoglutarate was found to be between 10^{-5} and 10^{-6}M. In such studies Fe^{++} could not be replaced by Cu^{+}. Ascorbic acid could be replaced by a number of ene-diol compounds but not by 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, NADPH or NADH. Addition of Na^{+} or K^{+} did not influence the reaction either in the presence or absence of α-ketoglutarate.

TABLE I

Requirements for Formation of Tritiated Water from Peptidyl-3,4-T-Proline Using Purified Chick Embryo Collagen Proline Hydroxylase

Complete Proline Hydroxylase System		Tritiated Water
Omissions	Additions	Formed (cpm)
None	None	720
α-Ketoglutarate	None	0
Ferrous ion	None	29
Ascorbic acid	None	0
Enzyme	None	0
α-Ketoglutarate	Dialysate	220
α-Ketoglutarate	Pyruvate	0
α-Ketoglutarate	Oxaloacetate	117
α-Ketoglutarate	Fumarate	0
α-Ketoglutarate	L-Glutamate	0

The complete system (2 ml) contained purified hydroxylase fraction (3 mg protein) and, in μmoles; ascorbic acid, 2.5; ferrous ion, 0.15; α-ketoglutarate, 0.02; Tris HCl, pH 7.5, 200; hydroxylase substrate, 800,000 dpm. The amount of dialysate added was equivalent to that obtained from 1.0 ml of S-105. In certain tubes α-ketoglutarate was replaced with 0.02 μmole of the indicated compound. Incubation was for 30 min at 30°.

To identify the activator in undialyzed enzyme preparations, these were preincubated with crystalline glutamic dehydrogenase, NADPH and NH_4Cl. As shown in Table II, such treatment destroyed all activity of the preparations. The activity of added α-ketoglutarate was also destroyed by such preincubation. To make certain that α-ketoglutarate did not in some way labilize tritium from the 3,4-T-proline residues, the resulting 3-T-hydroxyproline was isolated and shown to be formed in amounts equivalent to the tritiated water. In incubations containing the GDH system, the equilibrium concentration of α-ketoglutarate should have been less than 10^{-7} M, additional α-ketoglutarate having been aminated to form L-glutamate. The destruction by GDH of all activity in

TABLE II

Effect of Glutamic Dehydrogenase (GDH) on Collagen Proline Hydroxylase Activity in Crude Chick Embryo Extracts

Additions to Buffered Enzyme During Preincubation	Tritiated Water Formed (cpm)
None	815
GDH system minus GDH enzyme	752
GDH system complete	11
α-Ketoglutarate	1250
α-Ketoglutarate + complete GDH system	18

Undialyzed chick S-105 enzyme fraction (3 mg protein) was preincubated in a 1.0 ml vol containing 100 μmoles Tris HCl, pH 7.5, at 30°, 10 min. The GDH system, where added, contained 1.0 μmole NADPH; 50 μmoles NH_4Cl; GDH enzyme as described in the text. α-Ketoglutarate, where added, 0.10 μmole. At the end of the preincubation period, 1.0 ml of solution, containing 100 μmoles Tris HCl; 800,000 dpm hydroxylase substrate; 2.5 μmoles ascorbic acid and 0.15 μmole ferrous ion, was added to each tube. Final incubation was for 30 min at 30°.

undialyzed enzyme preparations and the ability of added α-ketoglutarate to replace the natural dialyzable activator are fairly good evidence that α-ketoglutarate is an absolute requirement for collagen proline hydroxylase. The low concentration required to stimulate *in vitro* activity is also compatible with a physiological role for this compound. Experiments similar to those shown in Tables I and II indicate that α-ketoglutarate is also required by collagen proline hydroxylase of rat skin and guinea pig granuloma.

It would appear that the hydroxylation reaction catalyzed by collagen proline hydroxylase utilizes ascorbate as the specific electron donating agent and iron as the electron transferring agent. Preliminary studies indicate that α-ketoglutarate dis-

appears during the reaction but that disappearance also occurs in the absence of Fe^{++} and ascorbate where hydroxylation does not occur. A likely explanation for the α-ketoglutarate requirement is that it is an allosteric activator of the enzyme. The tissue concentration of α-ketoglutarate is a good indicator of the availability of many amino acids and of the ATP generating capacity required for protein synthesis. Thus as an activator of proline hydroxylase, α-ketoglutarate could synchronize this critical step in collagen biosynthesis with the biochemical prerequisites of protein synthesis.

Acknowledgement: We wish to thank Dr. L.A. Fahien for suggesting the use of glutamic dehydrogenase in these experiments.

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